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Effects of iron chelates on embryogenic suspension cultures of carrot (Daucus carota L.)

Diane R. Shogren
Iowa State University

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by

Diane R. Shogren

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ABBREVIATIONS

DTPA	diethylenetriaminepentaacetic acid
EDDHA	ethylenediamine-di-(o-hydroxyphenol)-acetic acid
EDTA	ethylenediaminetetraacetic acid
MS	Murashige and Skoog tissue culture medium
PCV	packed cell volume
2,4-D	2,4-dichlorophenoxyacetic acid

LITERATURE REVIEW

Somatic Embryogenesis

Embryogenesis is the study of embryo development from its single-celled origin as a zygote to a mature embryo (Raghavan, 1986). An embryo is composed of an embryogenic axis and one or more cotyledons. The axis incorporates the root, the hypocotyl to which cotyledons are attached, and the shoot apex with the first true leaves (Bewley and Black, 1985). Somatic embryogenesis is a type of regeneration of embryo-like structures from somatic cells that closely follows the pattern of zygotic embryogenesis (Raghavan, 1986). Both zygotic and somatic embryos go through distinct stages of development: globular, heart-shaped, and torpedo-shaped stages. There are several recent reviews on the subject (Ammirato, 1983; Ammirato, 1987; Nomura and Komamine, 1986; Raghavan, 1983; Sharp et al., 1980; Williams and Maheswaran, 1986).

Much is known about embryogenesis in carrot (Daucus carota) because it is one of the most extensively studied systems in embryogenesis, but there is still much to be learned about embryogenesis. For example, it is still difficult to induce embryogenic development in monocots. Even among the most studied systems, little is known about embryogenesis at the molecular level, but new advances in techniques for studying gene expression are already illuminating the processes occurring as determination and development proceed in an embryogenic cell (Raghavan, 1986).

The process of obtaining a carrot cell suspension usually begins by starting callus cultures from seedling hypocotyls or root sections. The suspension is maintained in a tissue-culture medium such as MS (Murashige and Skoog, 1962). Hormones normally included are a cytokinin and an auxin, usually 2,4-D. To induce embryogenesis, the suspension is transferred to a medium without 2,4-D. Embryogenesis is usually evident four to five days later when cell aggregates in the suspension produce globular embryos. These globular embryos eventually develop into mature embryos which germinate (Raghavan, 1983).

A cultural factor having an effect on somatic embryogenesis in several plant species is the choice of iron chelate (Ammirato, 1983). Iron chelates have been shown to have an effect on growth and development of plant tissues, including the regulation of embryogenic development in vitro (Ammirato, 1983; Havranek and Vagera, 1979; Heberle-Bors, 1980; Raghavan, 1986; Sopory and Maheshwari, 1973; Vagera and Havranek, 1982a; Vagera and Havranek, 1982b; Vagera and Havranek, 1983).

Iron and Its Chelation in MS Tissue Culture Media

In MS, Nitsch, and many other plant tissue-culture media, ferrous sulfate chelated by EDTA is used as the iron source for tissue culture explants (Murashige and Skoog, 1962). The chelate is used to make iron available to the plant. If it were not present, the iron would precipitate becoming unavailable for use by the explant (Klein and Manos, 1960; Lindsay, 1974).

It has been noted that a precipitate appears in MS medium when the pH

is above 5, especially after autoclaving (Dalton et al., 1983). The precipitate consists mainly of ferric (Fe^{3+}) phosphate. The iron in MS is chelated by EDTA, which is an iron chelate of low stability when compared with DTPA and EDDHA. Because of the low stability of FeEDTA, other ions such as Ca^{2+} and Zn^{2+} can compete with iron for the chelate binding site. As a result, free unchelated iron in the medium is oxidized and precipitates with phosphate (Dalton et al., 1983; Lindsay, 1974). There are small quantities of other nutrients precipitated as well (Dalton et al., 1983; Vyskot and Bezdek, 1984).

Equilibrium of the chelate with a metal depends upon the competition between the metal and hydrogen ions for the chelate (Vyskot and Bezdek, 1984). The lower the pH at which the iron:chelate complex can exist, the lower the pH at which it dissociates. Trivalent iron forms a strong chelate with EDTA, dissociating from the chelate at a pH lower than 1, while bivalent iron (ferrous, Fe^{2+}) forms only a weak chelate, dissociating at pH 5. Precipitation can be alleviated by using a slightly lower ratio of iron to EDTA, such as 1:2 iron:EDTA. Also, the excess EDTA could complex with other metal ions that form more stable complexes with EDTA, preventing them from competing with iron for chelate binding sites allowing most of the iron to be chelated (Vyskot and Bezdek, 1984).

Bivalent iron is the form used in making MS medium (Vyskot and Bezdek, 1984). In MS medium 45% of the initial iron has been shown to precipitate in two days because of ineffective chelation (Dalton et al., 1983). Free bivalent iron at physiological pH (5.0-6.0) rapidly oxidizes to trivalent iron and precipitates to form ferric phosphate, a

form unavailable for plant use (Vyskot and Bezdek, 1984). Two ways were suggested by Dalton et al. (1983) to alleviate this problem. They were to lower the pH below 3.2 or increase the molar ratio of Fe:FeEDTA to from 1:1 to 1:3. Lowering the pH below 3.2 is not feasible in plant growth systems because nutrient availability is highly pH-dependent (Marschner et al., 1986), becoming either inadequate, adequate, or toxic depending upon the pH and the nutrient. Murashige and Skoog chose a pH range of 5.7-5.8 for their tissue culture medium. This pH range ensures that nutrients are in adequate supply (Murashige and Skoog, 1962). Raising the concentration of EDTA to three times the normal rate in MS (iron concentration remaining the same) resulted in lower growth rate and greening of Ocimum basilicum suspension cultures (Dalton et al., 1983). Using the normal rate of Na_2EDTA but a third of the normal rate of iron improved the greening rate of O. basilicum, and limited the packed cell volume, but did not change the dry weight (Dalton et al., 1983).

There are other iron chelates used in field situations that have also been used in tissue culture media to prevent precipitation. Examples of these alternative iron chelates are EDDHA and DTPA. These chelates are used by CIBA-GEIGY to chelate iron and are known as Sequestrene 138 and Sequestrene 330, respectively (Anonymous, 1986).

Effect of Iron Form on Androgenesis

In systems of Nicotiana, Datura, and Atropa, iron-free Nitsch medium can support embryogenic development of pollen grains in cultured anthers up to the globular stage, but further development of haploid embryos is

completed only in the presence of iron (Heberle-Bors, 1980; Nitsch, 1969; Raghavan, 1986; Sopory and Maheshwari, 1973; Vagera and Havranek, 1982a). In carrot, the lack of iron was used to stop embryo development at the globular stage, i.e., to "stage" the embryos (Vagera and Havranek, 1982a).

In another experiment, performed by Vagera and Havranek (1983), the effect of the form of iron on the development of androgenic plants from Nicotiana and Datura pollen grains was examined. They looked at the number of androgenic plants produced from the pollen grains in media with various forms of iron (bivalent or trivalent). Plant development was faster on the medium with only chelated trivalent iron compared to treatments on medium with free, unchelated trivalent iron or chelated iron in either the bivalent or trivalent form. The absence of trivalent iron enabled continuous development of embryos but no plants while its presence enabled development of embryos into plants but plant yield was lower than with bivalent iron (Vagera and Havranek, 1983). Development was slowest on medium with just EDTA and no iron. Embryos continuously developed on medium with unchelated iron or medium with no iron, but development did not proceed beyond the globular stage. Complete plants developed only in the presence of chelated iron (Vagera and Havranek, 1983).

Effect of Chelate Choice on Haploid Embryo Development

Choice of chelate, as well as the form of iron, has been shown to have an effect on growth and development. Heberle-Bors (1980) cultured anthers of Nicotiana and Atropa on Nitsch medium with different

chelates, including EDTA and EDDHA. Media with FeEDTA or FeEDDHA were tested for influence on post-androgenic development. Most normal embryos were in the heart-shaped or torpedo-shaped stage on the EDTA containing medium, while the embryos on the medium containing FeEDDHA remained at the globular stage (Heberle-Bors, 1980). FeEDTA gave the best response in terms of the number of haploid plants and also forms a less stable iron:chelate complex than FeEDDHA.

Of the three chelates, EDTA, DTPA, and EDDHA, EDTA forms the least stable complex with trivalent iron, DTPA forms the next least stable complex, and EDDHA forms the most stable complex with iron (Heberle-Bors, 1980; Norvell, 1972). The logarithms of the stability constants for these chelates are 25.1, 28.6, and 33.91 respectively (Chaberek and Martell, 1959; Chaney, 1988). The stability constant is the equilibrium constant for the reaction in which the metal chelate is formed from the hydrated metal ion and the most highly dissociated form of the chelating agent (Chaberek and Martell, 1959). The greater the stability constant, the greater the affinity the chelate has for a metal ion.

The effectiveness of the iron chelate in producing plants by embryogenesis seems to be inversely related to its stability, i.e., plants may not be able to utilize iron bound to a very stable complex (Heberle-Bors, 1980). In the Heberle-Bors experiment (1980), the FeEDDHA treatment resulting in embryogenic development was similar to that reported by Vagera and Havranek (1982a) for Nicotiana and Datura anthers cultured on medium without iron. Embryos developing from anthers in both cases did not develop beyond the globular stage. Another possible explanation for the response to FeEDDHA is that it

lowers the activity of trivalent iron when it is present in larger quantities. A greater concentration of FeEDDHA occurs as the plant tissue removes iron from the chelate. This leaves the chelate in the tissue culture medium at a greater concentration than iron. The result is iron deficiency because the free chelate accumulates depressing the activity of the free iron (Lindsay, 1974) as well as increasing the concentration of chelate with an ion other than iron bound to it (Marschner et al., 1986).

ABSTRACT

The influence of various chelates on cell suspension growth and somatic embryo development in carrot are described. The four treatments examined were: FeEDTA, 1/2FeEDTA, FeDTPA, and FeEDDHA. The 1/2FeEDTA treatment has half the iron concentration of the other three treatments. An inverse relationship was found between chelate stability and cell growth and embryo production. The FeEDDHA treatment, containing the most stable chelate examined, produced the lowest dry weight and the fewest embryos. The FeEDTA treatment, containing the least stable chelate examined, exhibited the greatest dry weight of the chelate treatments and produced the greatest number of embryos. The FeEDDHA and 1/2FeEDTA treatments were not significantly different from one another in producing a low number of embryos but did significantly differ in dry weight yield. The 1/2FeEDTA treatment was not significantly different from the FeEDTA treatment. During cell growth in the suspensions, iron is not limiting in the 1/2FeEDTA or FeEDDHA treatments, but during the rapid cell division occurring during embryo development iron becomes limiting in these treatments, stopping development at the globular stage.

INTRODUCTION

Iron chelates have been shown to have an effect on growth and development of embryos produced by androgenesis (Havranek and Vagera, 1979; Heberle-Bors, 1980; Sopory and Maheshwari, 1973). In work by Heberle-Bors (1980), anthers of Nicotiana and Datura were cultured on Nitsch media with different chelates, including EDTA and EDDHA. The ratio of normal to abortive embryos was similar on both media with either chelate, but on the medium containing FeEDTA most of the normal embryos were in the heart-shaped or torpedo-shaped stage while most of the embryos on the medium containing FeEDDHA remained at the globular stage. It was suggested by Heberle-Bors (1980) that EDDHA, being a high stability chelate, may have prevented iron uptake in the anther cultures. Nitsch noted that the removal of iron chelated by EDTA stopped androgenesis in Nicotiana at the globular stage (Nitsch, 1969), an observation which supports the suggestion of blocked iron uptake.

The effect of various chelates on somatic embryogenesis in in vitro cultures has not been examined although somatic cells would be expected to behave similarly compared with haploid cells. There has also been no attempt to look at the iron content of explants in tissue culture systems grown on media with different chelates or iron removal from these media by the explants.

The objectives of this research are to: 1.) find what effects chelates have on the growth of carrot somatic cell suspensions, 2.) see

if there is any regulation by the chelates of carrot embryo development and 3.) measure the iron content of the cells and the medium in which they were cultured.

MATERIALS AND METHODS

Seeds of Daucus carota L. 'Fanci Pak' were placed in a sidearm flask containing 0.5% NaOCl (10% v/v commercial laundry bleach) plus 0.02% Tween 20. The seeds were disinfested by aspirating and swirling continuously for 20 minutes. They were then rinsed three times with deionized water and transferred to a germination medium. Rinsing, transfer, and later subculturing were done under a laminar flow hood. The germination medium was MS basal medium composed of MS inorganic salts (Murashige and Skoog, 1962) to which were added (in mg/l): myo-inositol (100), thiamine-HCl (0.1), pyridoxine-HCl (0.5), nicotinic acid (0.5), glycine (2.0), sucrose (30 g/l), and Difco-Bacto agar (8 g/l). The pH was adjusted to 5.8 before adding the agar and the medium was autoclaved for 15 minutes at 121° C and 1.1 $kg \cdot cm^{-2}$.

The seeds were germinated in a dark growth chamber maintained at 24°C. After one week, the hypocotyls were cut into approximately 0.25 cm sections and transferred to basal MS with 0.5 $mg \cdot l^{-1}$ 2,4-D, 0.1 $mg \cdot l^{-1}$ kinetin (callus-induction medium), and one of four chelate treatments substituted for the 0.1 mM EDTA and 0.1 mM $FeSO_4 \cdot 7H_2O$. The four chelate treatment media consisted of MS callus induction medium with one of the following chelate and iron additions: 0.1 mM EDTA to 0.1 mM $FeSO_4 \cdot 7\ H_2O$, 0.1 mM EDTA to 0.05 mM $FeSO_4 \cdot 7\ H_2O$, 0.1 mM EDDHA to 0.1 mM $FeSO_4 \cdot 7\ H_2O$, or 0.1 mM DTPA to 0.1 mM $FeSO_4 \cdot 7\ H_2O$. Immediate placement on a treatment medium was done to prevent an iron:chelate carry-over effect which might be caused by the use of basal MS medium which contains iron chelated by EDTA. This carry-over effect was detected in preliminary

experiments.

Callus cultures were subcultured each month for three to five months before transfer to liquid culture. Callus was randomly selected from plates of each treatment medium and placed in the corresponding treatment medium in 125 ml straight-neck flasks. The flasks contained 30 ml of liquid basal MS with 0.5 $mg \cdot l^{-1}$ 2,4-D (without agar). The flasks were kept on orbital shakers at 100 rpm and 2.54 cm side-to-side stroke for one to four months in darkness at room temperature (19-21° C). The carrot cells were aseptically sieved through one layer of cheesecloth two to four weeks before the removal of 2,4-D to obtain a more homogenous-sized cell suspension.

The 2,4-D was removed from the suspensions to allow embryo maturation and germination by rinsing three times with hormone free liquid basal MS medium. Each rinse was removed by centrifuging at 138 relative centrifugal force (rcf)(1000 rpm, 12.3 cm radius) then removing the supernatant. After the third centrifugation the supernatant was removed, fresh liquid basal MS with the appropriate chelate was added, and the pellet resuspended. One-half ml PCV from each flask was added to 125 ml straight-neck flasks with 18 ml hormone-free MS medium. After two weeks 18 ml of fresh liquid basal MS medium (without 2,4-D) was added to the flasks. The cell suspensions remained on the shaker for another two weeks before they were removed. One-ml aliquots of suspension from each flask were removed and placed on solid basal MS treatment media (without hormones) to allow germination. The cultures were then placed in a lighted chamber at 25° C.

The following data were taken on the suspensions at the time the

flasks were removed from the shaker: PCV (experiments 1,2,3), fresh weight (experiment 2), dry weight (experiment 1), the number of embryos (experiments 1,2,3), the percent of abnormal embryos (experiments 1,2,3), iron concentration of the medium (experiments 2,3), and iron concentration of the cells (experiment 3).

The PCV was measured by centrifuging the contents of each flask in a graduated centrifuge tube and determining the mls of cell pellet. During this procedure, a drop of medium was removed from the supernatant and checked for bacterial contamination with crystal violet stain (29).

Fresh weight was taken by centrifuging cell suspensions at 138 rcf and the supernatant removed to 6ml (greater than the highest recorded PCV) after which the centrifuge tube and its contents were weighed. The cell suspension was then removed and rinsed three times with deionized water for iron measurement. The fresh weight was recorded as the difference in weight from the centrifuge tube with cell suspension and MS minus the weight of the same centrifuge tube with 6 mls MS.

Dry weight was taken by first weighing a Whatman paper filter. Then the cell suspension medium was poured through the filter leaving the cells on the filter paper. Five hundred mls of distilled water were poured through the filter after the suspension to remove sucrose from the filter. The filter with the cells was placed in a drying oven overnight at 80^{o} C., and weighed the following day. The initial filter weight was subtracted from the final weight to get the dry weight.

The total number of germinating and abnormal embryos were counted after two to three weeks under a stereoscope. Only the matured or germinating embryos were counted.

To determine the ppm of iron in the cell treatments, the cells had to be ashed. The cell suspensions were centrifuged and the supernatant was removed. Crucibles were weighed, then the cell pellet was placed in them. The cell suspension was dried in a drying oven at 80° C. for three days then placed in an ashing oven at 500° F overnight. Then 5 ml of 1:1 v/v HCl:distilled water was placed in each crucible. The contents of the crucible were then poured through a Whatman ashless filter paper. The filter was rinsed three times with hot distilled water. Finally, the cell rinse was diluted to a range (about 1 ppm Fe) that could be most accurately measured by an atomic absorbance spectrophotometer. This measurement was multiplied by the dilution factor and then expressed as micrograms of iron per ml PCV.

The experiment was repeated three times. As previously noted, not all data were taken for each experiment. Where possible, the data from experiments were combined for statistical analysis using SAS Proc GLM. The experimental design for each experiment was a randomized complete block design. Where appropriate, correlation and F-tests were applied to the data. The treatment sums of squares were divided into single degree-of-freedom F-tests by the use of non-orthogonal contrasts. The contrasts compared the following treatments: FeEDTA versus 1/2FeEDTA, and FeEDDHA versus 1/2FeEDTA. Correlation coefficients for the three chelate treatments were examined using SAS Proc CORR. Data from the 1/2FeEDTA treatment was deleted for this analysis. Only the treatments with the same molar ratios of iron were used because only differences among chelates and not levels of iron were desired to be examined.

The data for PCV of all three experiments was combined and analyzed,

and the data for ppm Fe in the second and third experiments were combined and analyzed. Date by treatment (date*trt) mean square for error was used to test the date, treatment, and linear effects sums of squares rather than pooled error because it was a better estimate of error. Date by treatment error measures error between experiments while pooled error measures the error within an experiment.

Dry weight was taken in the first and second experiments. The dry weight per flask was recorded for the first experiment while the combined dry weight of all the flasks for each treatment was recorded in the second experiment. The suspensions were combined at the end of the second experiment to ensure an adequate amount of plant material for measurable iron readings because a minimum concentration of iron must be present to be detected accurately. This was done because it was not known at the time of the second experiment if there would be enough iron in the suspension for a measurable reading. Only the dry weight from the first experiment was analyzed. Without replication in the second experiment, dry weight differences among treatments could not be tested.

In the third experiment, three flasks were randomly selected, combined, and ashed. The reason the flasks were combined rather than kept separate was to provide an adequate amount of cells for measurable iron readings and the combination of three flasks provided an adequate concentration.

The number of embryos and abnormals were recorded for each experiment but production was very poor in the second and third experiments so only the results from the first experiment were analyzed.

Iron in the culture medium was measured at the end of the culture

period when the cells were removed. Samples of treatment medium were diluted five fold in the following manner: 10 ml MS, 39 ml deionized water, and 1 ml v/v HCl:distilled water. These readings were taken for the second and third experiments only.

Iron in the cells was measured in each treatment from the second and third experiments and recorded as micrograms iron per ml PCV. The readings from the second experiment were not replicated so they were not statistically analyzed. In the third experiment, the cells of three flasks were randomly selected, combined, and analyzed.

RESULTS

There were significant differences among the four chelate treatments for embryo number (Table 1). The FeEDTA treatment, containing the chelate of the lowest stability (25.1), produced the most embryos per ml (4.44). The treatment with the chelate of intermediate stability (28.6), FeDTPA, produced fewer embryos per ml (2.66). The treatment with the most stable chelate, the FeEDDHA treatment (33.9), produced the fewest embryos per ml (0.45) (Figure 1). Correlation is a measure of the degree to which variables vary together or a measure of the intensity of association. When the correlation is small, the correlation coefficient (r) is near zero, if the coefficient is near 1 or -1 then the correlation is large. A negative coefficient indicates an inverse relationship among the two variables correlated (Steel and Torrie, 1980). The correlation coefficient for stability plotted against embryo number was -0.48 (Table 2) and this value was highly significant meaning the probability of a lesser or greater r value is less than 1%. Single degree-of-freedom F-tests indicated that the FeEDTA treatment had significantly more embryos than the 1/2FeEDTA treatment while the FeEDDHA and 1/2FeEDTA treatments were not significantly different (Table 1, Figure 1).

There were highly significant differences among the four chelate treatments for percent abnormal embryos (Table 3). The FeEDTA treatment had the second lowest percent abnormal embryos (17%) and the FeEDDHA had the greatest number (43%) (Figure 2). The correlation coefficient for stability plotted against the percent abnormal embryos was +0.52 and was

highly significant (Table 2). Single degree-of-freedom F-tests showed the FeEDTA treatment had significantly fewer percent of abnormal embryos than the 1/2FeEDTA treatment. The FeEDDHA and 1/2FeEDTA treatments did not differ significantly (Table 3, Figure 2).

There were highly significant differences among treatments for cell dry weight (Table 4). The 1/2FeEDTA treatment yielded the greatest dry weight (3.8 mg) while the FeEDDHA yielded the lowest dry weight (2.9 mg) (Figure 3). The correlation coefficient for stability plotted against dry weight was -0.43 and was significant (Table 2). Single degree-of-freedom F-tests show that the dry weight of cells in the FeEDDHA treatment was significantly lower than the dry weight of cells in the 1/2FeEDTA treatment. There was not a significant difference in dry weight between the FeEDTA and 1/2FeEDTA treatments (Table 4, Figure 3). There were also no significant differences among the FeEDTA versus 1/2FeEDTA and FeEDDHA versus 1/2FeEDTA treatments for the other growth measurements: PCV and fresh weight.

There were significant differences among the four treatments for concentration of iron left in the culture medium at the end of an experiment (Table 5). There was no apparent difference in the amount of iron left in the medium between the FeEDTA and FeEDDHA treaments (Figure 4). The correlation coefficient for stability plotted against iron concentration was +0.18 and was not significant (Table 2) which means there is no relationship among chelate stability constants and iron concentration of the medium. Significant differences appear in the single degree-of-freedom F-tests. There was significantly more iron left in the FeEDDHA treatment than the 1/2FeEDTA treatment at the end of

cell suspension culture period. As expected, the concentration of iron remaining in the FeEDTA treatment medium at the end of culture was significantly greater than the concentration remaining in the 1/2FeEDTA treatment medium (Table 5, Figure 4).

There were highly significant differences among the four treatments for micrograms iron per ml of PCV (Table 6). The correlation coefficient for stability plotted against iron per ml PCV was +0.14 and was not significant (Table 2). Significant treatment differences once again appeared in the single degree-of-freedom contrasts. There was a significant difference detected between the FeEDDHA and 1/2FeEDTA treatments for micrograms iron per ml PCV, the FeEDDHA treatment having significantly more iron. There was also significantly more iron per ml of PCV in the FeEDTA-treated cells compared with the 1/2FeEDTA treated cells (Table 6, Figure 5).

Table 1. Non-orthogonal single degree-of-freedom contrasts partitioning chelate treatment SS of the number of embryos per ml culture medium for the first experiment

Source	df	MS	F value
rep	11	8.85247	1.21
trt	3	34.06771	4.67**
FeEDTA vs 1/2FeEDTA	1	58.57881	8.03**
1/2FeEDTA vs FeEDDHA	1	2.60749	0.36NS
error	30	7.29723	
total	44	9.51130	

NS, ** Nonsignificant and significant at the 1% level, respectively.

Table 2. Pearson correlation coefficients (r) for chelate stability plotted against experimental measurements.

Measurements	r
embryos	-0.48^{**}
percent abnormal embryos	0.52^{**}
dry weight	-0.43^{*}
ppm iron	0.18^{NS}
iron per ml PCV	0.14^{NS}

^{NS}, *, ** Nonsignificant, significant at the 5% level, and significant at the 1% level, respectively.

Table 3. Non-orthogonal single degree-of-freedom contrasts partitioning chelate treatment SS of the number of abnormal embryos per ml of culture medium for the first experiment

Source	df	MS	F value
rep	11	0.021828	0.50
trt	3	0.379364	8.63^{**}
FeEDTA vs 1/2FeEDTA	1	0.479052	10.90^{**}
1/2FeEDTA vs FeEDDHA	1	0.004110	0.09^{NS}
error	26	0.043957	
total	40	0.063027	

^{NS}, ** Nonsignificant and significant at the 1% level, respectively.

Table 4. Non-orthogonal single degree-of-freedom contrasts partitioning chelate treatment SS of cell dry weight per ml culture medium for the first experiment

Source	df	MS	F value
rep	11	1.22529	1.26
trt	3	5.06875	5.20**
FeEDTA vs 1/2FeEDTA	1	2.83969	2.91NS
1/2FeEDTA vs FeEDDHA	1	14.95608	15.35**
error	30	0.97420	
total	44	1.31614	

NS, ** Nonsignificant, and significant at the 1% level, respectively.

Table 5. Non-orthogonal single-degree-of-freedom contrasts partitioning chelate treatment SS of ppm iron in the suspension medium at the end of the culture period for the second and third experiments

Source	df	MS	F value
date	1	0.001043	0.01
date(rep)	22	0.016058	0.91
trt	3	0.767515	10.88*
FeEDTA vs 1/2FeEDTA	1	1.576850	22.36*
1/2FeEDTA vs FeEDDHA	1	1.754808	24.88**
date*trt	3	0.070532	4.00*
pooled error	59	1.017629	
total	88	0.045516	

*, ** Significant at the 5% level and significant at the 1% level, respectively.

Table 6. Non-orthogonal single degree-of-freedom contrasts partitioning chelate treatment SS of micrograms iron per ml packed cell volume of cell suspension for the third experiment

Source	df	MS	F value
rep	3	0.022089	4.08
trt	3	0.089423	16.53**
FeEDTA vs 1/2FeEDTA	1	0.165728	30.64**
1/2FeEDTA vs FeEDDHA	1	0.163789	30.28**
error	7	0.005409	
total	13	0.028646	

** Significant at the 1% level.

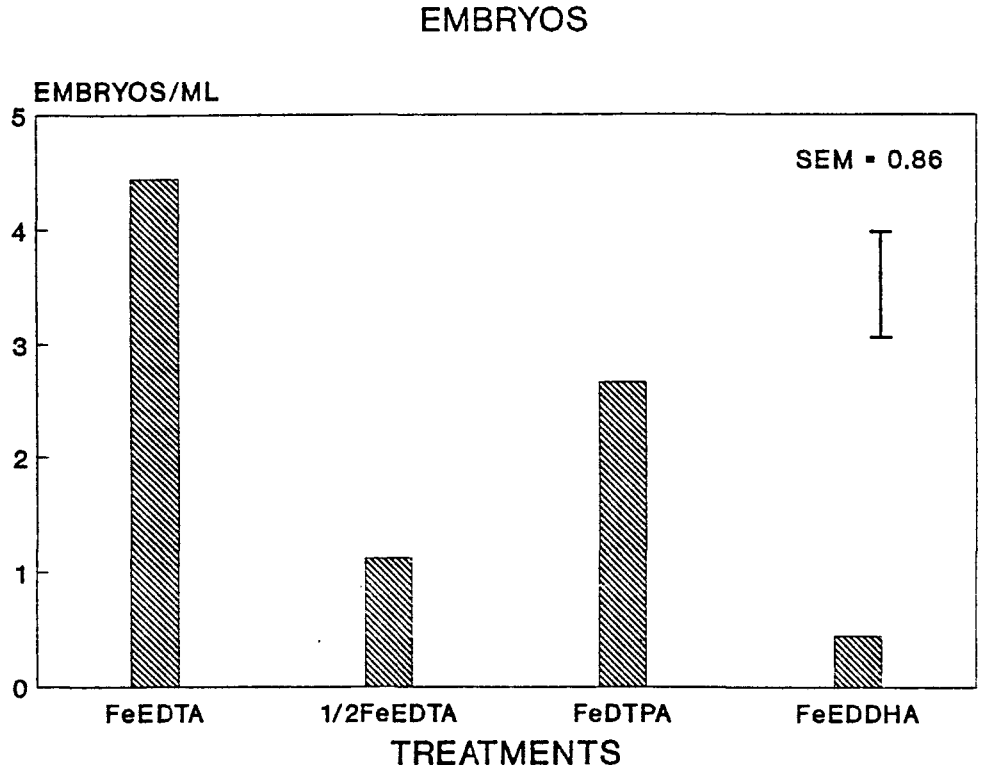

Figure 1. Mean and standard error of the mean for embryo number per ml of treatment medium for the first experiment. The chelates were present at a concentration of 0.1 mM and iron was present at 0.1 mM except in the 1/2FeEDTA treatment where iron was present at 0.05 mM

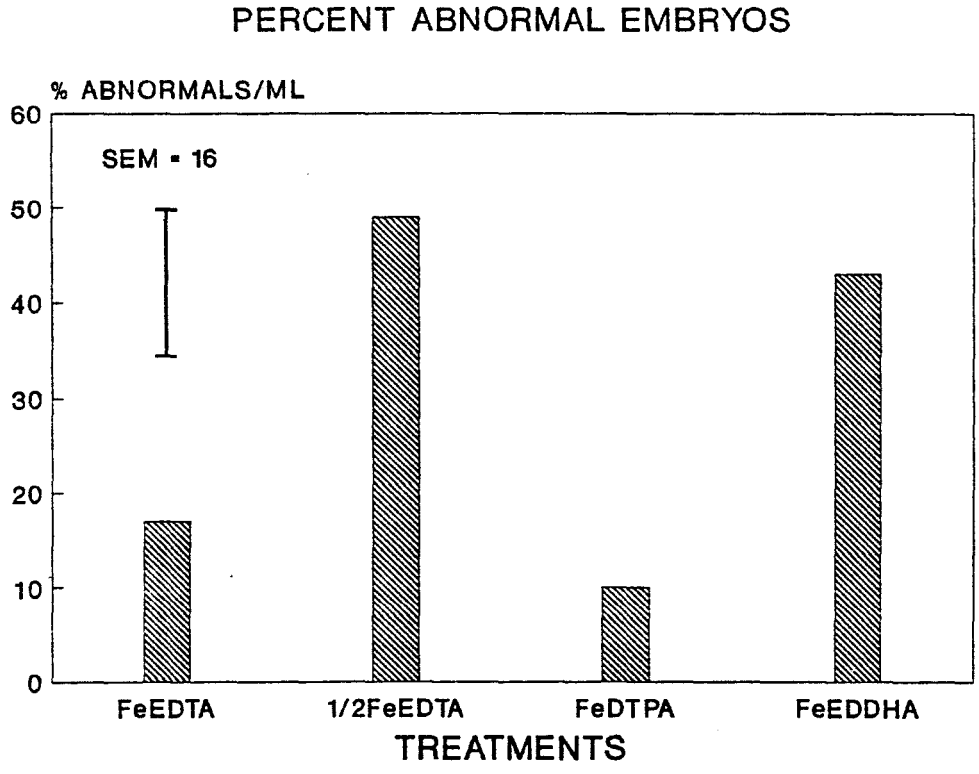

Figure 2. Mean percent and standard error of abnormal embryos per ml of treatment medium for experiment one. The chelates were present at a concentration of 0.1 mM and iron was present at 0.1 mM except in the 1/2FeEDTA treatment where iron was present at 0.05 mM

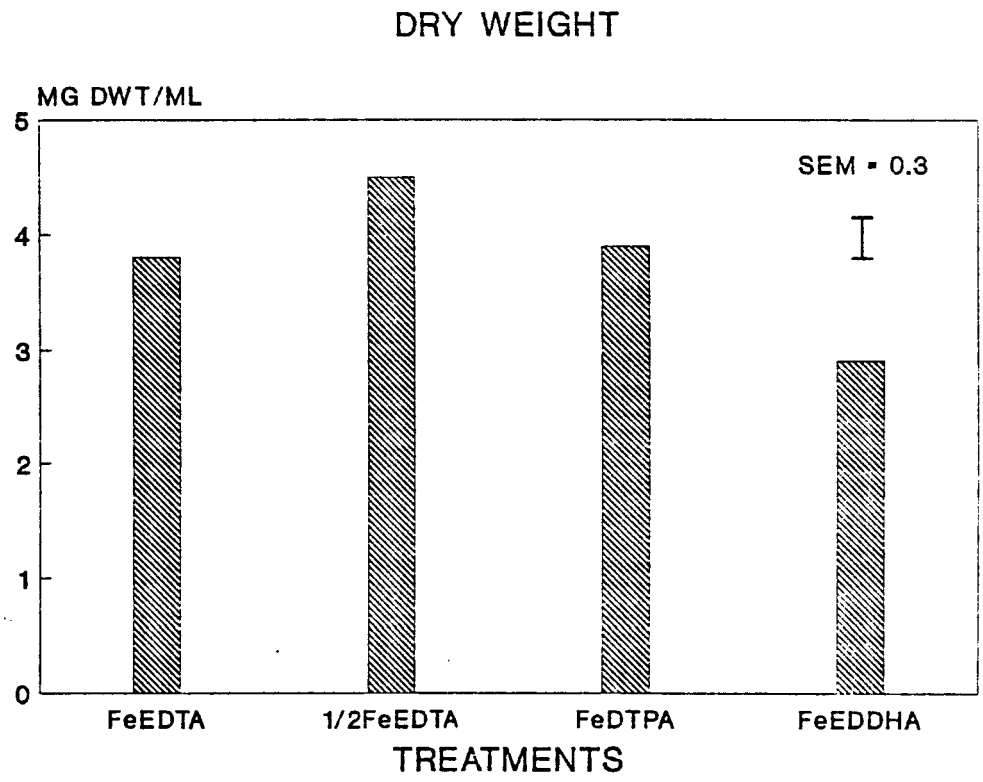

Figure 3. Mean and standard error of the mean for cell dry weight in mg per 100 ml of treatment medium for the first experiment. The chelates were present at a concentration of 0.1 mM and iron was present at 0.1 mM except in the 1/2FeEDTA treatment where iron was present at 0.05 mM

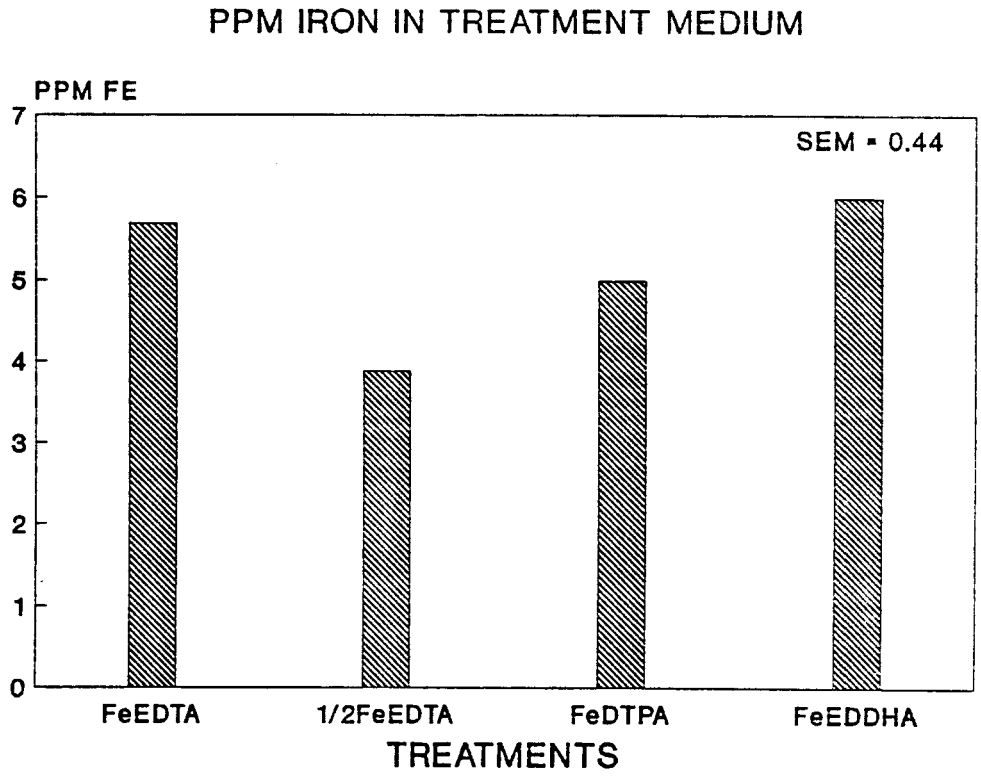

Figure 4. Mean ppm and standard error of the mean for iron in treatment medium samples taken at the end of the cell culture period in the second and third experiments. The chelates were present at a concentration of 0.1 mM and iron was present at 0.1 mM except in the 1/2FeEDTA treatment where iron was present at 0.05 mM

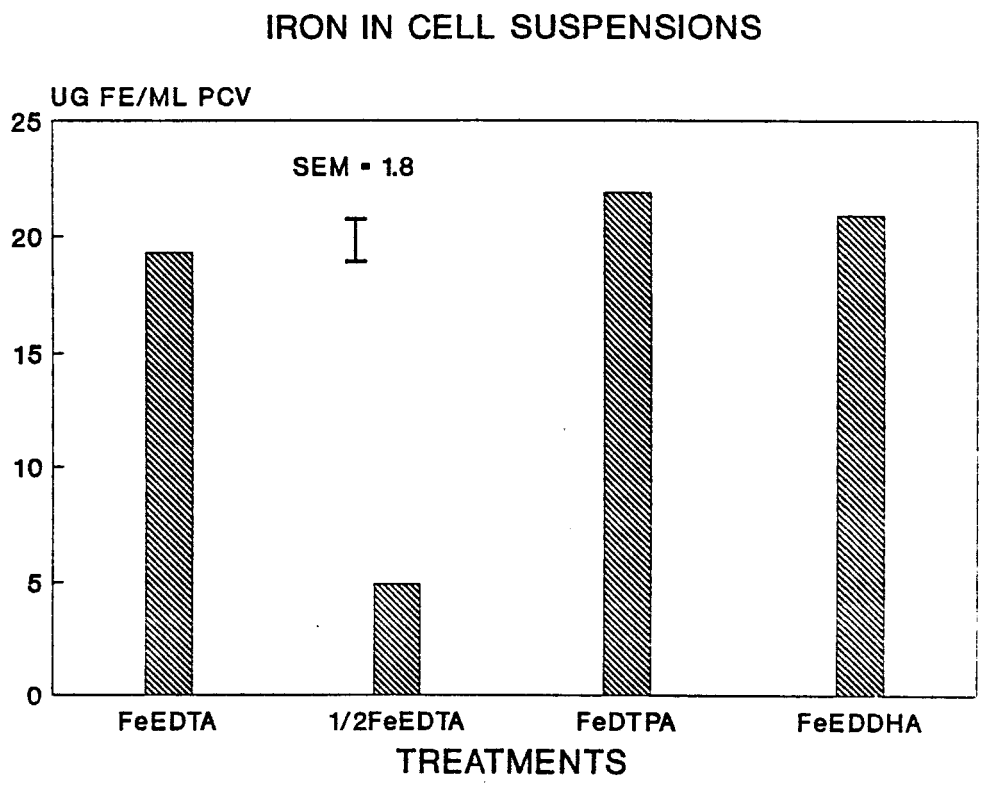

Figure 5. The mean ppm and standard error of the mean for micrograms iron per ml packed cell volume for the third experiment. The chelates were present at a concentration of 0.1 mM and iron was present at 0.1 mM except in the 1/2FeEDTA treatment where iron was present at 0.05 mM

DISCUSSION

As the chelate stability increases, the number of mature embryos produced decreases. EDDHA was the most stable chelate examined and the FeEDDHA treatment did not produce a significantly different number of mature embryos than did the 1/2FeEDTA treatment. Chelate stability and, therefore, iron availability (bound versus free iron) is dependent on many factors; medium pH, total iron concentration, and the concentration of ions competing for the chelate binding site are some of the most important. For example, it has been shown that the addition of plant cells into the culture medium and their subsequent growth changes pH (Street, 1977). These factors are continually changing during cell culture making it difficult to define exactly how much iron is in the free form and how much is bound by the chelate. The stability constants used here were for the ferric form of iron, because previous workers determined that the ferric form was predominant over the ferrous form in tissue culture medium where these three chelates were used (Dalton et al., 1983; Heberle-Bors, 1980; Vyskot and Bezdek, 1984). The ferric stability constants were used here as a convenient measure of the ratio of bound:free iron, even though it is recognized that this ratio may change under conditions discussed previously in this paragraph. Nevertheless, the order of chelate stability is not expected to change under these varying conditions. Therefore, linear correlation was used to describe the relationship between chelate stability and the experimental measurements of embryo number, percent abnormals, dry weight, ppm iron in the medium, and micrograms of iron per ml PCV. The

results suggest iron uptake by the embryos may be limited by the stability of the chelate just as iron concentration may have been limiting in the 1/2FeEDTA treatment though these differences were not detected with the atomic absorbance spectrophotometer. These results agree with the results of Heberle-Bors (1980). Androgenic embryos of Nicotiana and Atropa failed to develop beyond the globular stage in an FeEDDHA treatment but development continued in the FeEDTA treatment medium. Heberle-Bors hypothesized that this developmental regulation was a result of "severed" iron uptake caused by a very stable chelate:iron complex. Work by Nitsch (1969) showed that the lack of iron prevented haploid embryos of Nicotiana from developing beyond the globular stage. This work supports the hypothesis that the FeEDDHA and 1/2FeEDTA treatments are not providing enough iron for development of the somatic embryos beyond the globular stage.

The FeEDTA treatment had a significantly lower percent of abnormal embryos than the 1/2FeEDTA treatment. The 1/2FeEDTA and FeEDDHA treatments were not significantly different. The higher percentage of abnormal embryos found in these treatments with lower iron availability may be a reflection of iron deficiency. That is, not enough iron was present for normal embryo development.

The results for dry weight differ from the embryo results. In the case of dry weight, the 1/2FeEDTA treatment responded like the FeEDTA treatment rather than the FeEDDHA treatment. One reason may be that during normal cell division, enough iron is available for growth but, when embryogenesis is induced, iron is needed at a faster rate and becomes limiting because of rapid cell division during embryogenic

development. Fujimura and Komamine (1980) described three phases of cell division after auxin removal: 1.) 0-3 days when slow cell division occurred, 2.) 3-4 days when very rapid cell division occurred leading to the formation of a globular embryo, and 3.) 4-6 days where cell division occurred at a slower rate than the second stage because determination of differentiation had occurred. The cells are able to "pull" iron from the chelate before being embryogenically induced, but, when induced, the faster cell division leading to differentiation caused iron to become more limiting, stalling development at the globular stage. It would be interesting to compare the growth rates of suspensions grown in FeEDTA and FeEDDHA treatment medium to see if the logarithmic phase of growth after subculture to fresh medium would be different for each treatment. It would be expected, if iron was limiting during rapid cell division, that the growth phase in the FeEDDHA treated cells would occur later or not at all compared with the FeEDTA treatment growth phase.

To determine if iron was being removed in different amounts from the medium by different treatments, iron was measured in the culture medium at the end of the suspension culture period. The only difference found was among the iron concentration comparisons (1/2FeEDTA versus FeEDTA or FeEDDHA). This may be a result of inadequate machine sensitivity when iron concentration was measured, but this does show that differences seen among treatments are due to the effects of the chelate and not the concentration of iron in the medium.

The concentration of iron was also examined in the cells but, again, the only differences were found among the iron concentration comparisons. This may also be due to machine sensitivity. Possibly,

the cells may need a minimum amount of iron before dividing, so cell number and not cell iron content is affected when iron is limiting in cell suspensions.

There was almost no embryo production in the second and third experiments. A possible reason may be that the plant material was not cultured on the same time schedule before the removal of 2,4-D. This may have introduced variation in embryo response between experiments. The cultures used to start the hormone-free suspensions were eight and seven months old for the second and third experiments, respectively, compared to the five month old cultures used in the first experiment. The suspensions in the last two experiments may have passed their peak embryogenic potential by the time embryo development was induced (Smith and Street, 1973).

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